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by Kgs Ahmadi

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Modification of soy crude lecithin by partial enzymatic hydrolysis using phospholipase A1

¹Teti, E., ²Kgs. Ahmadi, ³Erliana, G. and ¹Priyanto, A. D.

¹Department of Food Science and Technology, Faculty of Agricultural Technology, Brawijaya University, Jl. Veteran, Malang, Indonesia 65141

²Department of Agroindustry Technology, Faculty of Agriculture, Tribhuwana Tunggal University, Jl. Telaga Warna, Tlogomas, Malang, Indonesia 65144
Indonesia Research Institute for Legumes and Tuber Crops (ILETRI), Kendalpayak, Malang, Indonesia

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Abstract

Lecithin or phospholipids is natural and widely used emulsifier. To improve its emulsifying properties, lecithin is modified by enzymatic hydrolysis using Phospholipase A1 (PLA1). PLA1 hydrolyzes at sn-1 position, that usually occupied by saturated fatty acids and more unsaturated fatty acid in lecithin is expected to increase hydrophilic properties. This study was aimed to investigate the hydrolysis time on the characteristics of partially hydrolyzed soy lecithin from Anjasmoro variety as one of Indonesian soybean superior varieties. The crude lecithin had hydrophilic-lipophilic balance (HLB) value of 5.0, and hydrolysis of Anjasmoro variety soybean lecithin by PLA1 increased HLB value due to increasing unsaturated fatty acids and lysophospholipids. PLA1 had a preference to hydrolyze saturated fatty acids. After hydrolysis, acetone insoluble (AI) of lecithin also increased because more polar lecithin was produced and phospholipids class was dominated by lysophospholipids. PLA1 is a promising enzyme for lecithin modification and might had advantage over PLA2 because of its action at sn-1 position that usually occupied by saturated fatty acids.

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Introduction

Lecithin or phospholipids are natural and widely used emulsifier in foods, pharmaceuticals, and cosmetics (Schneider, 2001). Commercial lecithin is derived from soybean and egg (Hara *et al.*, 2002). However, soy lecithin is preferred due to its low price and it is produced as by product of edible oil processing from water degumming step. Water degumming of soybean oil is low cost process, safe, applicable, and produces lecithin of 1.85-2.75% (Mounts *et al.*, 1996).

The molecular structure of phospholipids can be changed by either enzymatic or chemical means, and the aim of these processes is to obtain a tailor-made technological and/or physiological property that differs from the natural substrate. Enzymatic modification has gained increasing interest as enzymes can be used to modify phospholipids in a wide variety of ways (Vikbjerg *et al.*, 2006). Enzymatic modification is aimed to obtain more hydrophilic

lecithin (Colbert, 1998) with better oil-in-water emulsifying properties (Nieuwenhuyzen and Tomás, 2008). Partially hydrolyzed lecithin products possess improved emulsifying properties (Schneider, 1997) and the demand for hydrolyzed lecithin (lysolecithin) has been increasing in recent years (Cabezas *et al.*, 2011).

Enzymatically hydrolyzed lecithin has technological and commercial benefits since they are excellent oil-in-water (o/w) emulsifiers. The phospholipase A2 enzyme hydrolyzes specifically the fatty acid at the sn-2 position, which enables the processing of a range of lysophospholipids with different degrees of hydrolysis and emulsifying properties under controlled conditions. Different food applications or recipes will require the use of a lecithin with defined degree of hydrolysis and concentration. The modifications are essential for achieving and adjusting optimal ratios between hydrophilic and lipophilic properties and for ensuring good food processing ability (Nieuwenhuyzen and

*Corresponding author.
Email: teties@yahoo.co.id
teties@ub.ac.id

Tomás, 2008).

The availability of pure phospholipase A2, A1, and lipase enzymes now enables precise degrees of hydrolysis of the various phospholipids. Liquid products of hydrolyzed lecithin are increasingly used, and commercial use to a large extent is known only with phospholipase A2 (PLA2) for partial hydrolysis to produce lysophospholipids (Nieuwenhuyzen and Tomás, 2008) that more hydrophilic (Lilbæk *et al.*, 2007). PLA1 differs to PLA2 in hydrolyzed fatty acid position, as PLA1 hydrolyzes at sn-1 position (Bueschelberger, 2004), that usually occupied by more saturated fatty acids (Choo *et al.*, 2004). More unsaturated fatty acid in lecithin is expected to increase hydrophilic properties. Studies concerning to lecithin hydrolysis by PLA1 were sunflower (Goni *et al.*, 2010) and egg yolk (Vijeeta *et al.*, 2004) lecithin hydrolysis. Elimination of one molecule fatty acid from sn-1 position is expected to increase hydrophilic lipophilic balance to enhance the capability for stabilizing oil in water emulsion. Song *et al.* (2005) explained that PLA1 is not widely available, providing motivation in the search for various superior PLA1 sources for the industrial modification of phospholipids at the sn-1 position. An available stable PLA1 would overcome the low yield and conversion rates obtained with lipases.

The primary objective of current study is to investigate the hydrolysis time on the characteristics of partially hydrolyzed soy lecithin from Anjasmoro variety. Anjasmoro is one of superior soybean varieties developed in Indonesia that has high productivity and tolerant to puddle (Komariah, 2008).

Materials and Methods

Materials

Soybean var. Anjasmoro was obtained from Indonesia Research Institute for Legumes and Tuber Crops (ILETRI), Malang. Soybean oil was obtained by hydraulic pressing at 1.5×10^7 N/m² for 10 after heating the seed at 80°C for 30 min. Phospholipids standard (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylinositol) (Sigma Co.), phospholipase A1 or lecithase from *Thermomyces lanuginosus* with activity of 10 KLU/g (Sigma Co.), silica gel G60 TLC plate (Merck), standard mixture of FAME (Sigma Co.), and analytical grade chemical reagents from Merck.

Lecithin extraction by water degumming

Lecithin extraction was according to Eshratbadi (2008) with some modification. Lecithin was

extracted from soybean oil by water degumming. About 20 g \pm 0.1 mg was placed into beaker glass and 3% water was added and then the mixture was stirred and heated at 60°C for 30 min. Oil and water phase were separated by centrifugation at 300 rpm for 30 min. Gum or crude lecithin was formed in the lower layer (subnatant) and then dried in vacuum oven for 1 hour at 25°C and 1 atm.

Purification of crude lecithin

Purification was objected to reduce neutral oil from crude lecithin and this process was referred to method of Nasir *et al.* (2007). Oven dried crude lecithin was added by acetone in the ratio of crude lecithin to acetone of 1:6 (w/v) and the mixture was stirred for 1 hour. The solvent and lecithin was separated by decantation, and treatment was repeated until the solvent was colorless. Nitrogen was purged to discard residual solvent, then the lecithin purity was measured by thin layer chromatography (TLC).

Lecithin modification

About 5 g of purified lecithin was added by 10% (v/v solvent) water and stirred, then PLA1 (10% w/v solvent) was added to the mixture and 100 mL diethyl ether was poured. The mixture was incubated at 40°C for 10, 20, 30, 40, and 50 min, respectively. After incubation, the solvent was discarded and partially hydrolyzed lecithin was sprayed by nitrogen to eliminate residual solvent. Hydrolyzed lecithin was purified using acetone 1:4 to discard free fatty acids.

Analysis of phospholipids

Phospholipids analysis was performed using TLC according to method of Nzai and Proctor (1998) with slightly modification. About 1 mg of sample or standard was dissolved in chloroform:methanol (95:5) and then 10 μ L of sample or standard solution was spotted into TLC plate. TLC plate was activated by heating for 10 minutes at 100°C prior to analysis. The plates were developed for 40 minutes by chloroform:methanol:water 75:25:3 (v/v/v). Visualization was performed by charring using H₂SO₄:aquadest 1:1, and quantification by TLC scanner at 500 nm (Dual Wave Length Chromato Scanner CS-930 and Data Recorder DR-2 Shimadzu).

Fatty acid analysis

Fatty acid profile of lecithin before enzymatic hydrolysis was analyzed by gas chromatography (Shimadzu GC 8A). Column for separation was capillary CBP20 0.25 μ m bonded silica column with dimension of 50 m in length, i.d. 0.22 mm, o.d. 0.33 mm. Carrier gas was nitrogen (pressure of 200 kg/

m²), supporting gas was air (pressure of 0.15 kg/cm²), and burning gas was hydrogen with pressure of 0.6 kg/cm². Injector, column, and detector temperature was 230°C, 250°C, and 230°C, respectively. Samples and standard were injected in the volume of 2 µL. Methylation of phospholipids was performed according to Park and Goin (1994) method. Fatty acid composition of phospholipids was conducted after phospholipids separation by TLC. Each spot was scrapped off and extracted according to Christie (1982), while derivatization prior to GC analysis used Park and Goin (1994) method.

Data analysis

The treatment was replicated twice and the data was analyzed by analysis of variance and Least Significant Difference ($\alpha=0.05$). Acetone insoluble was determined according to method Ja 4-46 (AOCS, 2001), and HLB was determined based on saponification number (AOAC 920.160, 1996) and acid value according to method Ja 6-55 (AOCS, 2001).

Results and Discussion

Crude and purified lecithin

Lecithin from water degumming of Anjasmoro variety soybean oil had phospholipids content or purity of 57.90%, meanwhile purification by acetone washing produced lecithin purity of 83.96%. Lecithin purity is mainly determined by impurities content such as triglycerides and other nonpolar lipids. Triglycerides were dissolved in acetone, hence repeated acetone washing implied to removing of triglycerides that made increasing purity of lecithin.

For many industrial applications, crude lecithin products obtained from vegetable oil refining can be used directly. According to Vikbjerg *et al.* (2006), crude vegetable lecithin contains 30-40% neutral lipids, predominantly triglycerides. The remainder consists of polar lipids, mainly a mixture of different phospholipids (Vikbjerg *et al.*, 2006). Polar lipids (phospholipids and glycolipids) are almost insoluble in acetone (Bueschelberger, 2004).

To improve the handling of the highly viscous crude lecithin and to improve dispersability, industry commonly makes an acetone deoiling (Schneider, 1997). Triglycerides (TAGs) dissolve in acetone, in contrast to the other more polar components of lecithin. With acetone extraction, phospholipids become more concentrated, which results in significantly lower dosage requirements and higher functionality (Vikbjerg *et al.*, 2006). Food grade lecithin, produced by deoiling crude-grade lecithin

Table 1. Fatty acid composition of phospholipids classes of Anjasmoro variety soybean crude lecithin

Fatty Acid (%)	PI	PC	PE	PA
Lauric acid (C12:0)	7.39	nd	nd	3.09
Myristic acid (C14:0)	6.42	5.96	7.64	8.04
Palmitic acid (C16:0)	17.02	23.85	17.75	20.34
Palmitoleic acid (C16:1)	10.46	9.31	10.06	13.03
Stearic acid (C18:0)	7.60	6.73	nd	5.62
Oleic acid (C18:1)	29.85	5.99	10.06	nd
Linoleic acid (C18:2)	17.85	44.51	51.44	40.49
Linolenic acid (C18:3)	1.99	nd	nd	4.08
Arachidic acid (C20:0)	1.41	3.64	3.04	5.30

nd = not detectable

Table 2. Phospholipids composition of lecithin after hydrolysis using PLA1

Phospholipids Class (%)	Hydrolysis time (minutes)					
	0	10	20	30	40	50
Lysophosphatidylinositol (LPI)	nd	17.56	13.68	13.07	11.05	7.97
Lysophosphatidylcholine (LPC)	nd	16.79	21.48	24.45	28.66	30.14
Lysophosphatidylethanolamine (LPE)	nd	19.39	10.54	8.98	8.38	8.46
Phosphatidylinositol (PI)	15.1	10.70	5.76	4.69	3.20	nd
Phosphatidylcholine (PC)	26.1	1.71	21.23	1.73	21.55	11.20
Phosphatidylglycerol (PG)	nd	2.52	1.30	10.98	10.95	2.84
Phosphatidic acid (PA)	10.9	5.03	5.15	3.85	5.28	13.74
Phosphatidylethanolamine (PE)	5.8	3.72	4.63	18.29	nd	nd
Total phospholipids (PL)	57.9	23.68	38.07	39.54	40.98	27.78
Total lysophospholipids (LPL)	0	53.74	45.7	46.5	48.09	46.57
Purity (%)	57.9	77.42	83.77	86.04	89.07	74.35

nd = not detectable

via acetone extraction, contains 78% phospholipids (Hayes, 2004). In this study, repeated washing by acetone produced high purity lecithin with solid consistency and brownish color.

The crude lecithin had HLB value of 5.0, that suitable for water in oil (w/o) emulsions. Many food emulsion systems are oil in water (o/w) emulsions such as milk, mayonnaise, and coconut milk. The suitable HLB value for o/w emulsion is in the range of 8-18 (Belitz *et al.*, 2009). Partial hydrolysis was expected to make lecithin more hydrophilic that had higher HLB value.

Anjasmoro variety soy crude lecithin comprised of 26.1% phosphatidylcholine (PC), 5.8% phosphatidylethanolamine (PE), 15.1% phosphatidylinositol (PI), and 10.9% phosphatidic acid (PA). Wu and Wang (2004) reported that soy lecithin contained 18% PC, 14% PE, 9% PI, 2% PA, 2% other minor phospholipids, 11% glycolipids, 5% sugar complex, and 37% neutral lipid. Judde *et al.*

Table 3. Fatty acid composition of partially hydrolyzed lecithin

Fatty Acid (%)	Hydrolysis time (minutes)					
	0	10	20	30	40	50
Lauric acid (C12:0)	0.24	nd	nd	nd	nd	nd
Myristic acid (C14:0)	0.34	2.69	3.24	nd	nd	nd
Palmitic acid (C16:0)	26.00	17.86	18.40	21.89	21.52	16.51
Palmitoleic acid (C16:1)	0.23	4.64	3.68	7.87	4.42	6.56
Stearic acid (C18:0)	5.66	nd	nd	nd	nd	nd
Oleic acid (C18:1)	12.21	20.20	26.63	28.04	19.98	16.33
Linoleic acid (C18:2)	46.18	44.00	39.37	32.05	48.34	42.44
Linolenic acid (C18:3)	0.23	nd	nd	nd	nd	nd
Arachidic acid (C20:0)	4.36	4.59	4.06	nd	nd	nd
Behenic acid (C22:0)	2.25	nd	nd	nd	nd	nd
Eruic acid C22:1	1.21	6.03	4.61	10.16	5.74	18.15
Lignocenic acid C24:0	1.09	nd	nd	nd	nd	nd
Saturated	39.94	25.14	25.70	21.89	21.52	16.51
Unsaturated	60.06	74.86	74.30	78.11	78.48	83.49

nd = not detectable

Measured by relative percentage based on peak area of each fatty acid on the chromatogram

(2003) also reported that soy lecithins from various producers had different phospholipids composition. The differences between this study with that reported by Wu and Wang (2004), was due to the purity. In this study, sugar complex and neutral lipid was eliminated by acetone extraction that changed the phospholipids composition. It was supposed that soybean variety affect phospholipids composition, and the effect of soybean variety on phospholipids composition needs further study.

The composition of each phospholipids compound in lecithin was shown in Table 1. Previously, Wang and Wang (2008) reported that PC and PE as the main phospholipids class in soy lecithin had palmitic, stearic, oleic, linoleic, and linolenic acids with similar composition. PC and PE were dominated by linoleic acid. Our study showed that linoleic acid was the predominant fatty acid for PC, PE, and PA. However, PI was dominated by oleic acid. Different fatty acid composition might contribute to polarity of phospholipids class in lecithin.

Phospholipids composition of partially hydrolyzed crude lecithin

This study showed that partial enzymatic hydrolysis of purified lecithin led to phospholipids profile changes. After hydrolysis, even at 10 minutes hydrolysis time, lysophospholipids was detected (Table 2). Increasing hydrolysis time increased lysophospholipids compound in lecithin, except for hydrolysis time of 50 minutes. PLA1 has the ability to hydrolyze fatty acid at sn-1 position of phospholipids, producing free fatty acids and lysophospholipids, and the existence of lysophospholipids in lecithin was desired. Yang *et al.* (2008) described that the emulsifying capacity of lysophospholipids is higher than phospholipids for o/w emulsions, meanwhile glycerophosphorylic compounds are more hydrophilic. Song *et al.*, (2005) reported that lysophospholipids are excellent emulsifiers and

are particularly suitable for use in many industrial application such as food technology, cosmetics, and pharmaceutical industries.

Increasing hydrolysis time decreased phospholipids concentration, although the composition of phospholipids did not show regular pattern. It was indicated that PI tended to decrease, and the others (PC, PE, PA) changed irregularly. However, the purity of lecithin (indicated by total amount of phospholipids and lysophospholipids) increased in line to hydrolysis time. Hydrolysis of crude lecithin by PLA1 used diethyl ether as solvent medium, and liberated free fatty acids and neutral lipid in crude lecithin dissolved in the solvent that led to higher purity of lecithin.

Data in Table 1 showed that the major fatty acid of PI was oleic acid meanwhile PE, PC, and PA were dominated by linoleic acid. Hydrolysis by PLA1 increased oleic acid and tended to decrease linoleic acid (Table 3) on phospholipids structure. Possibly, PLA1 had preference to attack certain type of phospholipids hence fatty acid profile of phospholipids depended on type of phospholipids attacked and fatty acid liberated.

Hydrolysis of fatty acid from sn-1 position of phospholipids produced lysophospholipids. In the existence of water, further hydrolysis occurred that resulted in higher lysophospholipids and with possibility of glycerophosphorylic compounds formation. Wang (2001) noted that some PLA1s also possess lysoPLA activity, which removes the last fatty acid from lysophospholipids. Also, Richmond and Schmidt (2011) stated that it has been postulated for decades that sn-2 arachidonic acid (AA) cleavage from PLs may sometimes be mediated by concerted sequential PLA1/LysoPLA2 activities, thus in this study possibly led to produce glycerophosphorylic compound. Glycerophosphorylic compound did not measure in this study because it could not be separated by TLC and supposed not to move in TLC plate due to its polar characteristic. Glycerophosphorylic compound formation was supposed to make irregular concentration changes in some phospholipids compounds (PC, PA, PE).

Acyl migration from sn-1 to sn-3 in phospholipids structures are possible to occur during hydrolysis by PLA1. Yang *et al.* (2008) explained that hydrolysis by PLA1 had the mechanisms as followed: (1) The fatty acid at the sn-1 position of phospholipids is hydrolyzed by phospholipase A1; (2) spontaneous acyl migration takes place with the fatty acids at the sn-2 position moving to the sn-1 position; (3) phospholipids are hydrolyzed to produce lysophospholipids and glycerophosphorylic compounds.

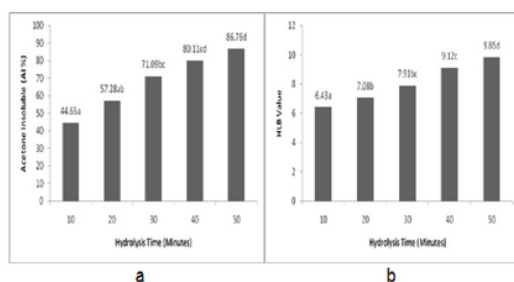


Figure 1. Effect of hydrolysis time by PLA1 on acetone insoluble (a) and HLB value (b) of lecithin. Means followed by letters (a, b, c, etc.) are significantly different at $\alpha=0.05$.

PLA1 is an enzyme that has the ability to hydrolyze and synthesize ester linkage in phospholipids structure. Acyl group-modifying PLA1 attack the carboxylic ester bonds of phospholipids, thus removing and replacing the acyl chain in various phospholipids via the reaction mechanisms of hydrolysis, esterification, and transesterification. Song *et al.* (2005) noted that water and enzyme concentration determined whether hydrolysis or esterification action of PLA1. Hydrolysis time of 50 minutes decreased the purity of lecithin (Table 1), and this was due to sharp decline in phospholipids content. Water was probably still available for long time hydrolysis, and that hydrolysis still occurred even for 50 minutes hydrolysis. It was supposed that reesterification of liberated free fatty into lysophospholipids structure was inhibited by the availability of water. Excessive amount of water (10%) was added for lecithin hydrolysis, and it was supposed that glycerophosphorylic compounds might form that could decrease the purity of lecithin.

Fatty acid composition of partially hydrolyzed crude lecithin

Linoleic acid was predominant fatty acid of Anjasmoro variety soybean lecithin, and some studies reported by Das and Bhattacharyya (2006); Wang and Wang, (2008) showed that the major fatty acid in soy lecithin was linoleic acid. Soy lecithin was dominated by unsaturated fatty acid that was a typical of vegetable lecithin. Lecithin hydrolysis by PLA1 could also changed the composition of soy lecithin (Table 3).

Increasing hydrolysis time decreased saturated fatty acid, meanwhile unsaturated increased (Table 3). This phenomena was in accordance with the action of PLA1 to hydrolyze sn-1 position that occupied by saturated fatty acids. Among unsaturated fatty acids, linoleic acid tended to decrease, but other unsaturated fatty acids increased. PLA1 was supposed to have preference for hydrolyzing palmitic acid and in some extent linoleic acid. According to Mounts *et al.*

(1996), sn-1 position in lecithin was dominated by linoleic and palmitic acid and they comprised 47% and 24% of fatty acids in that position.

Acetone insoluble of partially hydrolyzed crude lecithin

The often used quality characteristic acetone-insoluble (AI) matter includes phospholipids, glycolipids, and carbohydrates all together. The amount of AI matter (% AI) is the approximate indication for the amount of phospholipids, glycolipids, and carbohydrates, because the oil and fatty acids dissolve in acetone (Nieuwenhuyzen and Tomás, 2008). Hydrolysis time affected acetone insoluble of partially hydrolyzed lecithin. Crude lecithin before hydrolysis had AI of 66.36%, and hydrolysis time of 10 minutes decreased AI of lecithin (Figure 1a). Hydrolysis by PLA1 liberated fatty acid from sn-1 position of phospholipids that resulted in more hydrophilic lecithin due to elimination of non polar fatty acyl moiety from phospholipids molecules. Free fatty acids dissolved in acetone that made AI decrease, while further increasing hydrolysis time led to increase AI. Crude lecithin was dissolved in diethyl ether during hydrolysis that might contribute to the increasing AI. Some dirt in lecithin, such as neutral lipid and free fatty acid, would dissolve in non polar solvent.

The compositional changes of fatty acids attached to phospholipids molecules during hydrolysis might alter phospholipids polarity. Data in Table 2 showed that increasing hydrolysis time caused more unsaturated fatty acids in phospholipids. Unsaturated fatty acids are more hydrophilic than saturated ones that more unsaturated fatty acids in phospholipids structure led to increasing hydrophilic property of phospholipids. Hence, hydrolysis produced lysophospholipids containing more unsaturated fatty acids compared to original phospholipids that led to more hydrophilic phospholipids production. Conversion of phospholipids into lysophospholipids, as shown in Table 3, also changed the polarity of lecithin. Therefore, lysophospholipids was more polar than phospholipids that contribute to insolubility in acetone.

Hydrophilic-lipophilic balance of partially hydrolyzed crude lecithin

The modifications of lecithins are essential for achieving and adjusting optimal ratios between hydrophilic and lipophilic properties and for ensuring good food processing ability (Nieuwenhuyzen and Tomás, 2008). HLB is important parameter to measure the suitability of lecithin with food products. An HLB

system is often used for the selection of emulsifiers and is a measure of the surfactant's preference for oil or water, with the higher the number corresponding to a greater hydrophilicity-to-lipophilicity ratio (Vikbjerg *et al.*, 2006).

Crude soy lecithin from Anjasmoro variety had HLB of 5.0, and this HLB was influenced by phospholipids class in lecithin as well as fatty acid composition. If there are more unsaturated fatty acid, lecithin was supposed to be more hydrophilic. Vikbjerg *et al.* (2006) showed that particle sizes in emulsion were shown to be influenced by the length and degrees of unsaturation of the acyl chain of the PC.

HLB value increased with increasing hydrolysis time (Figure 1b), and this value was affected by fatty acid saturation. Therefore, increasing unsaturated fatty acids due to increasing hydrolysis time contributed to HLB value. PLA1 hydrolyzed fatty acid at sn-1 position that usually occupied by saturated fatty acid, hence this caused fatty acid to be more unsaturated that enhance HLB value. Phospholipids composition might contribute to HLB value, and conversion of phospholipids into lysophospholipids due to the action of PLA1 (Table 2) caused more hydrophilic phospholipids composition.

Conclusion

Hydrolysis of Anjasmoro variety soybean lecithin by PLA1 increased HLB value due to increasing unsaturated fatty acids and lysophospholipids. Acetone insoluble of lecithin also increased because lecithin was more insoluble in acetone due to more polar lecithin was produced after hydrolysis. PLA1 is a promising enzyme for lecithin modification and might had advantage over PLA2 because of its action at sn-1 position that usually occupied by saturated fatty acids.

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